NEW ANTIVIRAL ANTIBIOTICS; XANTHOCILLIN X MONO- AND DIMETHYLETHER, AND METHOXY-XANTHOCILLIN X DIMETHYLETHER. II

BIOLOGICAL ASPECTS OF ANTIVIRAL ACTIVITY

(STUDIES ON ANTIVIRAL AND ANTITUMOR ANTIBIOTICS. VI)

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Effects of XME, XDE and XTE (xanthocillin X mono- and dimethylether, and methoxy-xanthocillin X dimethylether) on multiplication of Newcastle disease virus (NDV) and on proliferation of chick embryo fibroblast cells (CEF) were examined. At low multiplicity, XME, XDE and XTE suppressed CPE at low concentrations. At high multiplicity, XME (10 mcg/ml) completely inhibited occurrence of CPE and syntheses of HA and infective virus, and at lower concentrations there was no difference in final yields of HA at 23 hours post infection, but a concentration dependency was found in synthesis of HA and suppression of CPE. XDE and XTE, on the other hand, did not inhibit HA synthesis completely at high concentration, but delayed the onset of HA production and partially arrested the occurrence of CPE post infection. All three antibiotics had very low toxicity against both grown and growing primary CEF. They also had no effect on free NDV particle and on viral adsorption onto CEF.

Searching for antiviral antibiotics, we found acetone extract of two strains of soil fungi exerted remarkable antiviral activity against Newcastle disease virus (NDV) and vaccinia and herpes simplex viruses in agar diffusion-plaque inhibition method, and isolated the active principles in crystalline form. They were identified as xanthocillin X mono- and dimethylether, and methoxy-xanthocillin X dimethylether (XME, XDE and XTE, respectively)[1,2].

The three antibiotics had low cytotoxicity against primary culture of chick embryo fibroblast cells (CEF) at higher concentrations than that required for suppression of occurrence of cytopathic effect (CPE) following viral growth. Their antiviral activity was studied employing NDV and primary culture of CEF virus-cell system. The present paper reports the effect on cell growth, on free virus particle and a adsorption of NDV onto CEF and also the antiviral activity determined by tube assay method of these antibiotics.

Materials and Methods

Virus and Cell:

NDV Miyadera strain and primary monolayer culture of CEF system was used; proli-

feration of virus and cell was the same as that described previously[8] except that Tris-HCl buffered (0.005 M, pH 7.4) medium was employed.

Antibiotics:

Recrystallized XME, XDE and XTE[2] were used for the present experiment.

Infection by NDV:

Confluent monolayer of CEF in test tube (10×200 mm) or Petri dish (90 mm in diameter) was washed twice with precooled (5°C) medium and infected by NDV at multiplicities indicated in each experiment. After 2-hour incubation at 5°C, unadsorbed virus was removed by washing twice with prewarmed (38.5°C) medium, and cell sheet was refed with the medium and incubated at 38.5°C.

Measurement of Cell Growth:

For the determination of action of the antibiotics on CEF growth, monodispersed CEF prepared from 8~10 day-old embryos by treatment with trypsin (1:250, Difco Laboratories, Michigan, U.S.A.) was suspended in Tris-buffered medium at 5×10^5 cells/ml, distributed evenly into test tubes (10×200 mm), and antibiotic was added to duplicate tubes at each concentration. Rubber-capped tubes containing 1 ml cell suspension were incubated at 38.5°C for 3 days; cell growth was surveyed under direct microscopic observation and was expressed by the degree of confluency of cell sheet as follows: −, no cell attachment; +, 1~25 % confluency; ++, 26~50 % confluency; +++, 51~75 % confluency; and ++++, 76~100 % confluency.

Inactivating Effect on Free NDV Particle:

NDV was suspended in medium containing XME, XDE or XTE and was incubated at 38.5°C. At various times aliquots were sampled and residual infective virus was determined by counting plaque forming unit (PFU) on monolayer of CEF.

Effect on Viral Adsorption onto CEF:

NDV was added to cell suspension in antibiotic-containing medium, and the virus cell suspension was allowed to stand at 5°C with occasional stirring. At various times, 1 ml was centrifuged at 1,000 rpm for 10 minutes to spin down viruses adsorbed onto CEF, and residual PFU in supernatant medium was counted and expressed in percentage of input PFU.

Measurement of Viral Growth:

Multiplication of NDV in CEF was followed by measuring production of infective virus or of hemagglutinin (HA) in duplicate tubes and was expressed in PFU or hemagglutinin unit (HAU), respectively. In both cases, medium and cells were frozen and thawed three times in a dryice-acetone bath, and PFU and/or HAU was titrated.

Hemagglutinin Titration:

HAU was determined by pattern method after addition of an equal volume of 0.2 % red blood cells prepared from adult hen. For more accuracy, the degree of hemagglutination reaction was expressed as +, ++−, +−, +−− and −, and multiplication by the factor of 2, $2^{\frac{3}{4}}$, $2^{\frac{1}{2}}$, $2^{\frac{1}{4}}$ and 1, respectively, was done.

Results

(1) Antiviral Activity in Tube Assay Method

The antiviral activity of XME, XDE and XTE on NDV multiplication was examined at two levels of input multiplicity of infection, *i.e.*, at 1 and 50 PFU/cell. When CEF monolayer in tube was infected at multiplicity of 1 PFU/cell and was treated with the antibiotics after 2-hour adsorption period at 5°C, CPE after 2 days viral growth was suppressed by XME, XDE and XTE at concentrations of 1.25, 4.15 and 5.0 mcg/ml, respectively (Table 1). Next, the input multiplicity was increased to

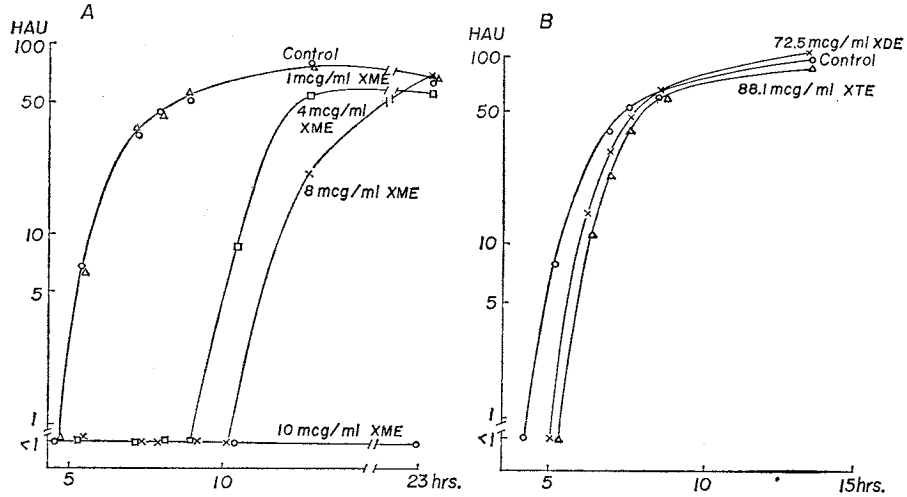
Table 1. Dose response of the antiviral activity of xanthocillin X mono- and dimethylether, and methoxy-xanthocillin X dimethylether against NDV determined by tube assay method

Xanthocillin X monomethylether		Xanthocillin X dimethylether		Methoxy-xanthocillin X dimethylether	
Conc. (mcg/ml)	CPE	Conc. (mcg/ml)	CPE	Conc. (mcg/ml)	CPE
10.0	—	8.30	—	10.0	—
5.0	—	4.15	—	5.0	—
2.5	—	2.08	+++	2.5	+
1.25	—	1.04	++++	1.25	+++
0.63	+	0.52	++++	0.63	++++
0.31	+++	0 (Control)	++++	0.31	++++
0 (Control)	++++			0 (Control)	++++

CEF monolayer in test tube prepared from 9~10 day old embryos were infected by NDV Miyadera strain at multiplicity of 1 before confluency. After about 40-hour incubation at 38.5°C, degree of cytopathic effect (CPE) by NDV was surveyed under direct microscopic observation and was expressed as follows; — : no CPE, + : 1~25 % cells showed CPE, ++ : 26~50 % cells showed CPE, +++ : 51~75 % cells showed CPE, and ++++ : 76~100 % cells showed CPE.

Fig. 1. Dose-response of the inhibitory effect of xanthocillin X mono-, dimethylether and methoxy-xanthocillin X dimethylether on one step growth of NDV.

CEF was infected at input multiplicity of 50 PFU/cell and antibiotics were added at 0 time. Duplicate tubes of each concentration were frozen and thawed three times, and viral yield was expressed in HAU.



50 PFU/cell and viral growth was followed by hemagglutinin titration in addition to microscopic survey of occurrence of CPE. As shown in Fig. 1 A, XME inhibited completely the production of HA at 10 mcg/ml, and a dose-response was observed in the length of lag time before detectable synthesis of HA at less than 10 mcg/ml, but at 1 mcg/ml there was no difference in kinetics of HA production.

In the case of XDE and XTE no complete inhibition of HA synthesis was detected even at 72.5 and 88.1 mcg/ml, respectively, at which concentrations some turbidity was observed because of their slight solubility in water, but at these concentrations the lag time in HA synthesis was lengthened as shown in Fig. 1 B. The final yield of

HA at 23 hours post infection was the same as that in the control when infected cells were treated with XME at concentrations of 8, 4 or 1 mcg/ml, but the degree of CPE was less in comparison with that of the control where cell rounding and complete destruction of cell sheet were observed, and dependency on antibiotic conentration was also shown in degree of suppression of CPE. The same was found with XDE and XTE, but the suppression was less than with XME.

In the above experiments, NDV growth was followed by HAU titration and/or microscopic survey of CPE, but the possibility could not be neglected that HA synthesis and/or CPE progressed in the absence of production of infective virus. To examine this possibility, HAU and PFU at 15 hours post infection were compared at various concentrations of antibiotics (Table 2). Arrest of HA production was found to nearly parallel the inhibition of infective virus multiplication at concentrations tested.

Table 2. Effect of XME, XDE and XTE on synthesis of infective NDV and of hemagglutinin

Antibiotic	Concentration (mcg/ml)	% PFU	% HAU
Control		100	100
XME	16.0	<1.0	<1.0
	8.0	<1.0	<1.0
	4.0	28.0	35.0
	2.0	89.6	100
	1.0	103.3	100
	0.5	94.6	100
	0.25	101.0	100
XDE	60.0	81.4	82.5
	30.0	78.0	82.5
	15.0	93.9	100
	7.5	105.3	100
	3.75	98.1	100
XTE	60.0	29.3	42.5
	30.0	75.4	70.5
	15.0	73.5	82.5
	7.5	112	100
	3.75	92.4	100

Monolayer culture of CEF in test tube was infected by NDV at input multiplicity of infection of 50 PFU/cell, and antibiotic was added after 2-hour adsorption period. PFU and HAU were determined at 15 hours after infection and were expressed as % of control.

Table 3. Effect of xanthocillin X mono- and dimethylether, and methoxy-xanthocillin X dimethylether on growth of chick embryo fibroblast cells

Concentration (mcg/ml)	Xanthocillin X monomethylether	Xanthocillin X dimethylether	Methoxy-xanthocillin X dimethylether
10.0	+	++++	+++
5.0	++	++++	++++
2.5	++++	++++	++++
1.25	++++	++++	++++
0 (Control)	++++	++++	++++

Duplicate tubes containing 1 ml cell suspension (5×10^5 cells/ml) at each antibiotic concentration were incubated at 38.5°C for 3 days and cell growth was measured by direct microscopic observation. The degree of cell growth was expressed as follows :

— : no growth
+ : 1~25 % confluency
++ : 26~50 % confluency
+++ : 51~75 % confluency
++++ : 76~100 % confluency

(2) Effect on CEF Growth

At concentrations employed above, no cytotoxic effect caused by the antibiotics was detected under direct microscopic survey. To examine the effect on growth of CEP, freshly prepared monodispersed cells were incubated in the presence of one of the three antibiotics and cell sheet formation was observed after 3-day incubation at 38.5°C. As indicated in Table 3, no difference was shown in cell sheet confluency between antibiotic-treated and controls at concentrations of 2.5, 10 and 5.0 mcg/ml, respectivly,

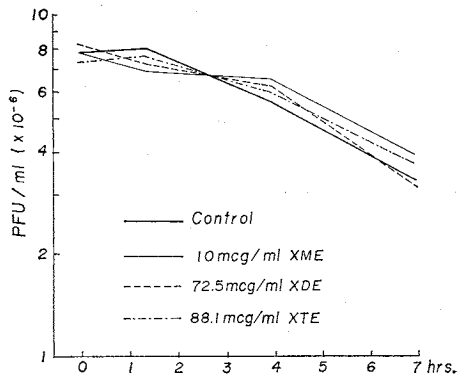
of XME, XDE and XTE. Some proliferation of CEF was observed even at 10 mcg/ml XME at which concentration it arrested completely HA production and occurrence of CPE (Fig. 1 A). The results shown in Table 3 indicate that all the three antibiotics had very low cytotoxicity against both grown and growing cultured cells.

(3) Inactivation of Free NDV Particles

As predicted from the results that antibiotics had low toxicity against CEF, they also had no inactivating effect on free NDV particles at 38.5°C and the velocity of decrease of residual PFU was the same in control and antibiotic-treated at higher concenttrations than required for inhibition of viral growth (Fig. 2).

Fig. 2. Inactivating activity of xanthocillin X mono-, dimethylether and methoxy-xanthocillin X dimethylether on free NDV particles at 38.5°C

NDV suspension was incubated at 38.5°C in the presence or absence of antibiotics. At times indicated, residual infectivity was determined by counting PFU on CEF monolayer.



(4) Viral Adsorption onto CEF

All three antibiotics did not affect the adsorption of NDV onto CEF, and residual PFU in supernatant after contrifugation was the same in the presence and absence of the antibiotics (Table 4).

Table 4. Effcet of xanthocillin X mono- and dimethylether, and methoxy-xanthocillin X dimethylether on NDV adsorption onto CEF

Antibiotic	Concentration (mcg/ml)	Residual PFU (% of input PFU)
XME	20	17.6
XDE	72.5	16.3
XTE	88.1	18.0
Control	——	17.3

NDV was added to cell suspension in Tris-HCl buffered medium containing one of the antibiotics at the concentration indicated in the table, and virus-cell suspension was allowed to stand at 5°C with occasional stirring. Residual PFU was measured after removal of adsorbed NDV by centrifugation and was expressed in % of input PFU.

Discussion

At low input multiplicity of infection (< 1 PFU/cell), XME, XDE and XTE showed remarkable antiviral activity in both plate and tube assay methods (Fig. 1 and reference 2), but only XME arrested completely the viral growth when multiplicity was increased to 50 PFU/cell.

A dose-response was detected in the length of lag time before onset of HA synthesis when infected cells were treated with XME. Once HA synthesis began, no difference was observed in velocity and final yield (Fig. 1). XME may affect some early events in HA synthesis, but clarification is needed.

All three antibiotics had no effect on free NDV particles (Fig. 2) and on viral absorption onto host cells, and also good correlation was found between HA production and infective virus multiplication (Table 2). From these results, it may safely be concluded that these antibiotics inhibit some event(s) following viral adsorption and before maturation in viral one-step growth cycle. This was confirmed by further study with XME[4].

References

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